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Short communication

Detection of *Babesia bigemina* infection in strains of *Rhipicephalus* (*Boophilus*) *microplus* collected from outbreaks in South Texas

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Abstract

The sudden death of several cattle infested experimentally with *Rhipicephalus* (*Boophilus*) *microplus* led to a clinical investigation into the reasons for the unexpected mortality. Microscopic evidence for *Babesia bigemina* infection was found in blood smears from the affected animals and a PCR assay was designed to detect the presence of *B. bigemina* and *Babesia bovis* in all *R. microplus* strains received and propagated at the laboratory. The assay utilizes a nested PCR approach with the first PCR amplifying a well-conserved segment from the *Babesia* 18S ribosomal RNA gene followed by a nested PCR with *Babesia* species-specific primers and annealing temperatures enabling amplification of the 18S ribosomal RNA gene fragment specific to either *B. bigemina* or *B. bovis*. DNA from groups of 50 larvae was extracted using a rapid DNA preparation protocol, which consisted of grinding the frozen tick larvae in PCR buffer and boiling the mixture for 5 min. The assay sensitivity allowed for the detection of the equivalent of a single infected tick larva. *R. microplus* eggs were also analyzed, but yolk protein viscosity created inconsistent results with the crush and boil DNA isolation protocol, necessitating the use of a more extensive proteinase K digestion-based DNA purification method. We detected the presence of *B. bigemina* in all strains of *R. microplus* currently reared at the laboratory and 4 of 26 strains collected from infestation outbreaks in Texas by the U.S. Cattle Fever Tick Eradication Program.
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1. Introduction

Babesiosis is a serious disease of cattle caused by protozoan parasites of the genus *Babesia*. The disease is transmitted during blood feeding by infected ticks and is the most economically important arthropod-transmitted agricultural disease in the world (Bock et al., 2004). The severity of the economic impact of babesiosis to Southern U.S. cattle producers in the 19th and 20th centuries led the U.S. Department of Agriculture to initiate a coordinated federal-state eradication program aimed at the disease vector *Rhipicephalus microplus* and *Rhipicephalus annulatus* ticks (Graham and Hourrigan, 1977). Although the eradication program was declared completed in 1943, *R. microplus* and *R. annulatus* remained widespread in Mexico and incidents of tick-infested cattle along the Texas–Mexico border are a constant threat to reintroduce babesiosis to the U.S. (Bram et al., 2002).

Recently at the U.S. Cattle Fever Tick Research Laboratory (CFTRL), following infestation of newly obtained cattle with *R. microplus*, several animals became severely ill and blood tests revealed low levels of infection with *Babesia bigemina*. Several strains of *R. microplus* and *R. annulatus* are reared at the laboratory

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for research purposes and in support of the U.S. Cattle Fever Tick Eradication Program. Some of these tick strains originated from collaborations with colleagues in Mexico. Also, the CFTRL receives and develops strains, termed outbreak strains, from ticks collected at sites of *R. microplus* infestation outbreaks occurring in the Tick Eradication Quarantine Area along the Texas–Mexico border. It became necessary to test each tick strain for the presence of *Babesia* and the literature contained several publications of PCR-based assays for detecting *Babesia* in either cattle blood (Figueroa et al., 1993; Calder et al., 1996) or ticks (Oliveira-Sequeira et al., 2005; Hartelt et al., 2004). We have designed and verified the performance of a two-step nested PCR assay that utilizes a primary PCR to amplify template DNA molecules from conserved regions of the *Babesia* 18S ribosomal RNA gene followed by a secondary nested PCR which specifically amplifies either *B. bigemina* or *B. bovis* 18S template, depending on primer choice. We screened each CFTRL tick strain for the presence of the two major *Babesia* species responsible for cattle babesiosis in Mexico, *B. bovis* and *B. bigemina*. We also obtained data on the infection status of 26 *R. microplus* infestation outbreaks, which were detected and eradicated by personnel from the U.S. Cattle Fever Tick Eradication Program.

2. Materials and methods

2.1. Ticks

Ticks were reared on cattle at the CFTRL following procedures described by Davey et al. (1980). Larvae or eggs were collected in centrifuge tubes while still viable and immediately frozen at −80 °C. Tick material was kept frozen until just before grinding for DNA extraction. Table 1 describes the origin location and date of each tick strain and the generation tested for *Babesia* infection. The *R. annulatus* San Ambrosia tick strain had been in the laboratory less than a year prior to the observed *B. bigemina* infection outbreak and had been reared in relative isolation from the numerous *R. microplus* tick strains. San Ambrosia was expected, and eventually verified, to be *Babesia*-free and would serve as a negative control and also to verify that commensual microorganisms present on *R. microplus* ticks did not amplify under the PCR assay conditions. Larvae and eggs from the *B. bovis*-infected Pullman strain of *R. microplus* were obtained from the USDA-ARS laboratory (Pullman, WA) and utilized as a positive control sample for *B. bovis* infection and negative control sample for *B. bigemina*. Larvae and eggs from the Munoz tick strain generation f28 served as positive controls for *B. bigemina* infection, as these ticks had been used in the experimental infestation of calves, which were subsequently clinically diagnosed as infected with *B. bigemina*. As *B. bovis* infection was not detected in the *B. bigemina*-infected calves, the Munoz f28 larvae served as a *B. bovis* negative control sample. For routine diagnostic analysis of the samples reported in this study, a clump of larvae or eggs approximately 3 mm diameter was used for DNA purifications. By not counting out individuals, considerable time was saved when analyzing large numbers of samples and, despite the variations inherent in numbers of individuals per each ‘‘ approximately 3 mm diameter clump’’ (200–300 individuals), assay performance was not affected. For determination of assay sensitivity using tick material as template, 1, 5, 10 and 50 eggs or larvae were aliquoted on dry ice to prechilled microcentrifuge tubes prior to crushing for DNA extraction.

2.2. DNA preparation

Genomic DNA was prepared by either a rapid grind-boil method (Czank, 1996) or a more extensive proteinase K-based method (Ballinger-Crabtree et al., 1992). Both methods utilized disposable pellet pestle grinders (Kontes, Vineland, NJ) prechilled in liquid nitrogen to pulverize the tick material in microcentrifuge tubes chilled in dry ice. For the proteinase K method, the pulverized material was resuspended in 300 μl of buffer (100 mM Tris–HCl pH 8.0, 50 mM NaCl, 50 mM EDTA, 1% sodium dodecyl sulfate, 0.15 mM spermine, 0.5 mM spermidine) and 5 μl of a 20 mg/ml proteinase K solution, followed by overnight digestion at 50 °C, two extractions with 25:24:1 phenol:chloroform:isoamyl alcohol, ethanol precipitation, and resuspension in 150 μl of 10 mM Tris–HCl pH 8.0. Phase Lock Gel (Eppendorf, Hamburg Germany) was utilized according to the manufacturer’s instructions to facilitate the extractions. For the crush-boil method, the pulverized material was quickly resuspended in buffer (100 mM Tris–HCl pH 8.3, 500 mM KCl) and immediately boiled for 3–5 min. The quantity of buffer was 50 μl when extracting DNA from pools of 25 or more individuals (eggs or larvae) and 25 μl for 10 or fewer individuals.

2.3. PCR

Twenty microliter reactions contained 10 mM Tris(hydroxymethyl)aminomethane hydrochloride pH

Table 1
Tick strains evaluated by nested PCR for *Babesia bigemina* or *Babesia bovis* infection

Name	Source	Received in Lab	Material tested			PCR result	
			Stage	Generation		*B. bigemina*	*B. bovis*
				No.	Date		
R. annulatus							
San Ambrosia[a]	Webb Co., TX outbreak	11/12/04	Larvae	f4	9/05	–	–
			Eggs	f5	9/05	–	–
R. microplus							
Clareno	Outbreak	1/25/98	Larvae	f1	1/98	+	–
Coatzacoalcos[a]	Coatzacoalcos, Mexico	8/8/94	Larvae	f12	7/96	–	–
			Larvae	f54	9/05	+	–
			Eggs	f55	9/05	+	–
Deutch[a]	Webb Co., TX outbreak	10/16/01	Larvae	f1	12/01	+	–
			Larvae	f16	9/05	+	–
			Eggs	f17	9/05	+	–
Las Palmas	Zapata Co., TX outbreak	3/22/05	Larvae	f1	9/05	+	–
Munoz[a]	Zapata Co., TX outbreak	8/22/99	Larvae	f28	4/05	+	–
			Larvae	f29	9/05	+	–
			Eggs	f30	9/05	+	–
Pesqueria[a]	Pesqueria, Mexico	1/4/01	Larvae	f21	9/05	+	–
			Eggs	f22	10/05	+	–
San Alfonso[a]	Tabasco, Mexico	10/29/02	Larvae	f20	9/05	+	–
			Eggs	f21	9/05	+	–
San Felipe[a]	Soto la Marina, Mexico	7/6/95	Larvae	f48	9/05	+	–
			Eggs	f49	9/05	+	–
San Roman[a]	Champoton, Mexico	8/15/98	Larvae	f36	9/05	+	–
			Eggs	f37	9/05	+	–
Santa Luiza[a]	Santa Luiza, Brazil	9/15/00	Larvae	f23	10/05	+	–
			Eggs	f24	9/05	+	–
Tuxpan	Tuxpan, Mexico	8/94	Larvae	f16	10/97	+	–
Valmar	Outbreak	6/12/02	Larvae	f1	6/02	+	–
B. bovis-infected *R. microplus* strain							
Pullman	USDA-ARS lab strain	5/10/04	Larvae	ND[b]	5/04	–	+

[a] Strain routinely maintained in CFTRL laboratory.
[b] ND: no data.

8.3, 50 mM KCl, 0.05 mM each dNTP, 1 μM each primer, 1.75 mM $MgCl_2$, and 0.1 μl of a 1:1 (v/v) mix of *AmpliTaq* DNA Polymerase (5 units/μl stock; Applied Biosystems, Foster City, CA) and TaqStart Antibody (1.1 μg/μl stock; Clontech, Palo Alto, CA). One microliter of template DNA was used for the primary PCRs, both undiluted and diluted 1:5 or 1:50 in H_2O with two replicates of each template concentration. The nested PCRs utilized 0.5 μl of primary PCR product mix as template. Amplification for the primary PCR was carried out using a DNA Engine (MJ Research, Watertown, MA) programmed for 95 °C for 2 min followed by 40 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 66 °C for 1 min, and extension at 72 °C for 1 min. The program also included a final extension step at 72 °C for 7 min. The thermocycling conditions for the *B. bovis* nested PCR was identical to the primary PCR except the annealing step was only 30 s. The thermocycling conditions for the nested *B. bigemina* PCR differed from the primary PCR conditions only in the annealing step (69 °C for 30 s). Reaction products were fractionated on 4% agarose TBE gels and DNA was visualized by staining with GelStar DNA Staining Dye (FMC Bioproducts, Rockland, ME) followed by UV illumination. The sizes of the diagnostic PCR products in the nested PCR for *B. bigemina* (GenBank accession no. AY603402) and *B. bovis* (GenBank accession No. L19078) were calculated to be 262, and 217 bp, respectively. To verify the identity of these diagnostic PCR products and the

specificity of the protocol, bands migrating at 262 and 217 bp were cut from agarose gels, cloned into pPCR-Script Amp SK+ (Stratagene, La Jolla, CA) and sequenced. As additional positive controls for PCR, we used *B. bigemina* and *B. bovis* 18S gene fragments cloned into pPCR-Script Amp SK+. High purity plasmid preparations were quantified by absorbance spectroscopy and serial dilution used to prepare plasmid solutions of 1 ng/μl–0.01 fg/μl. One microliter of each dilution was used as template to examine sensitivity of the PCR assay with purified template. As a further evaluation of the protocol's usefulness in a diagnostic laboratory setting, a blind study was designed using standardized DNA prepared from larvae of the uninfected San Ambrosia strain of *R. annulatus*, the *B. bigemina*-infected Munoz strain of *R. microplus*, or eggs from the *B. bovis*-infected Pullman strain of *R. microplus*. Templates were assayed by two individuals who performed both the *B. bigemina* and *B. bovis* PCR assays on several replicates of each template using template aliquots identified only by numerals. To test the assay's ability to accurately detect mixed infections, samples containing equal amounts of DNA prepared from both the *B. bigemina*-infected Munoz ticks and the *B. bovis*-infected Pullman eggs were included. Each blind study was performed using two replicates each of undiluted and 1:5 diluted numbered template.

3. Results

3.1. Development of assays

The quick crush-boil DNA extraction procedure worked well on larval samples. However, it gave unacceptably high false negative results with egg samples possibly due to the extreme viscosity of the denatured egg proteins following boiling. Thus to reliably analyze egg samples, we found the longer proteinase K protocol to be necessary. Figs. 1 and 2 demonstrate the extreme sensitivity of the nested PCR assay as 1 fg of positive control plasmid added to serve as template in the primary PCR produces an easily detectable product in agarose electrophoretic analysis of products from the nested PCR protocol. One femtogram of plasmid template in our PCR assay is the equivalent of approximately 40 copies of the rRNA template. Dalrymple (1990) reported *B. bovis* contains three copies of the rRNA gene and this would convert to a detection capability of approximately 14-parasite genome copies in the 1 μl volume of DNA template utilized in the assay. The analysis of gel-extracted DNA from the diagnostic bands which migrated in agarose gels at 262 and 217 bp showed the sequences were identical to the predicted 18S gene fragments from *B. bigemina* and *B. bovis*, respectively. In the blind study, two individuals assayed eight samples using two replicates each of undiluted and 1:5 diluted templates. The template was scored as positive for infection if any replicate produced a diagnostic band following gel analysis. Each individual correctly identified the *B. bigemina*/*B. bovis* infection-status of all assayed templates and each dilution replicate pair gave consistent results (data not shown).

Representative gel electrophoresis results for the *B. bigemina* assays are shown in Fig. 1. The 262 bp diagnostic band is easily visible in the nested PCR product from assays utilizing undiluted template (lane 3) or 1:50 diluted template (Lane 5) from pools of 50 larvae or template prepared from a single larvae (Lane 8) using the quick crush-boil procedure. One femtogram of *B. bigemina* 18S control plasmid consistently

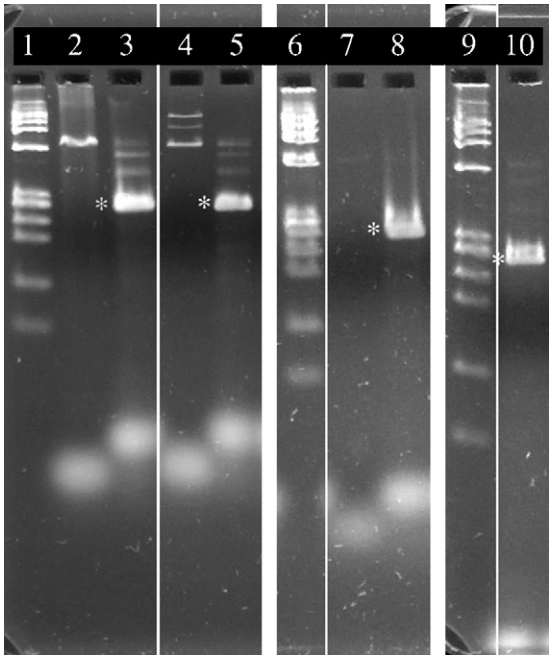

Fig. 1. Agarose gel analysis of representative *B. bigemina* PCR assays. Three different experiments are represented in lanes 1–5 (50 larvae pooled), 6–8 (single larva) and 9–10 (positive control plasmid). Lane identification: (1), (6), and (9); ϕX *Hae* III digest DNA markers (Invitrogen, Carlsbad, CA); (2) primary PCR products from undiluted DNA template; (3) nested PCR products from undiluted DNA template; (4) primary PCR products from 1:50 diluted DNA template; (5) nested PCR products from 1:50 diluted DNA template; (7) primary PCR products from undiluted DNA template; (8) nested PCR products from undiluted DNA template; (10) product from nested PCR using 1 fg of positive control plasmid; (*) denotes 262 bp diagnostic DNA band.

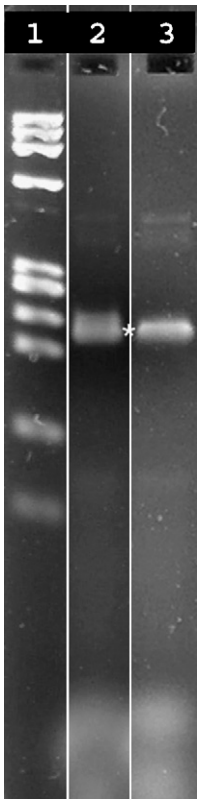

Fig. 2. Agarose gel analysis of representative *B. bovis* PCR assays. Two different experiments with the Pullman strain are represented in lanes 1–2 (50 larvae pooled) and 3 (positive control plasmid). Lane identification: (1) ϕX *Hae* III digest DNA markers (Invitrogen, Carlsbad, CA); (2) nested PCR products from 1:50 diluted DNA template; (3) product from nested PCR using 1 fg of positive control plasmid; (*) denotes 217 bp diagnostic DNA band.

produced sufficient product to be easily visualized (Lane 10). Representative gel electrophoresis results for the *B. bovis* assays are shown in Fig. 2. The 217 bp diagnostic band is easily visible in the nested PCR product from assays utilizing 1:50 diluted template (Lane 2) from pools of 50 larvae. One femtogram of *B. bovis* 18S control plasmid consistently produced a visible diagnostic product band (Lane 3) while 0.01 fg produced a visible amplification product in only 1 of 30 replicates (data not shown).

3.2. Analysis of tick strains

The PCR assay results are shown in Table 1. Only the Pullman *B. bovis*-infected strain of *R. microplus* tested positive for *B. bovis*. All of the *R. microplus* strains routinely maintained in the laboratory were positive for *B. bigemina* infection while the only *B. annulatus* strain maintained in the laboratory was negative for both species of *Babesia*. Additionally, in every case where an egg sample was tested along with the preceding generation's larval sample, both samples gave consistent results. For example, the San Ambrosia f4 larvae and the f5 eggs were both negative for *B. bigemina* and *B. bovis*, while the Deutch f16 larvae and f17 egg samples were both negative for *B. bovis* and positive for *B. bigemina*. Using archival material from the Coatzacoalcos, Deutch, and Munoz tick strains, we discovered that the *B. bigemina* infection of these strains was present for many generations prior to the observed cattle host infection, which prompted this study (data not shown). The Deutch strain came to the laboratory from a *R. microplus* outbreak on a Texas ranch and the larval sample designated as f1 was obtained from eggs of 55 engorged female ticks collected from the ranch. The females were not placed on laboratory animals prior to oviposition, thus, the *B. bigemina* infection detected by our assay was obtained in nature and brought into the lab with the tick sample. The Clareno, Las Palmas, and Valmar outbreak strains also tested positive for *B. bigemina* in the f1 generation larval sample, which had not been placed on laboratory host animals, again indicating that ticks sampled from infestation outbreaks in Texas were infected with *B. bigemina*. Larvae or eggs from 22 other infestation outbreaks were tested and found to be *Babesia*-free (data not shown).

4. Discussion

Using the primers described in Table 2 and DNA prepared from *B. bigemina*-infected larvae from the Munoz strain and the Pullman *B. bovis*-infected tick strain, we designed a nested PCR to efficiently detect *B. bigemina* and/or *B. bovis* in *R. microplus* or *R. annulatus*. Our target life stages were the larvae and eggs as these are most easily sampled and assayed during laboratory rearing protocols. Our objective was to design a relaxed-sensitivity primary PCR which would amplify DNA from any *Babesia* species in our samples followed by a subsequent stringent nested PCR to strictly amplify DNA from either *B. bigemina* or *B. bovis*, depending on choice of primers. Prior to designing our own assays, we considered protocols in the literature. Hartelt et al. (2004) designed a PCR assay to detect *Babesia* sp. in *Ixodes ricinus* ticks. However they reported difficulties with the specificity of their 18S primers and our BlastN analysis of their primers showed insufficient specificity to meet our need to specifically identify ticks harboring *B. bigemina* or *B. bovis*. Calder et al. (1996) used PCR to assay for *B.*

Table 2
Sequences of PCR primers

Primer ID	Sequence	Description	Annealing site
Primary PCR			
KB-16	5′-CAT-CAG-CTT-GAC-GGT-AGG-G-3′	Upstream primer	nt 275–293[a], 257–275[b]
KB-17	5′-GTC-CTT-GGC-AAA-TGC-TTT-C-3′	Downstream primer	nt 838–856[a], 804–822[b]
Secondary (nested) PCRs			
B. bigemina			
KB-18	5′-GAT-GTA-CAA-CCT-CAC-CAG-AGT-ACC-3′	Upstream primer	nt 478–501[a]
KB-19	5′-CAA-CAA-AAT-AGA-ACC-AAG-GTC-CTA-C-3′	Downstream primer	nt 715–739[a]
B. bovis			
KB-24	5′-GGG-GGC-GAC-CTT-CAC-3′	Upstream primer	nt 455–469[a]
KB-25	5′-CTC-AAT-TAT-ACA-GGC-GAA-AC-3′	Downstream primer	nt 652–671[b]

[a] Nucleotide corresponding to *B. bigemina* accession no. AY603402.
[b] Nucleotide corresponding to *B. bovis* accession no. L19078.

bovis infection in cattle blood samples. However, BlastN analysis of their primer sequences revealed sequence mismatches which might lead to detection difficulties and an unacceptable level of false negatives. Additionally, their assay would not detect *B. bigemina* as the sequences in their primer binding region are quite different in *B. bigemina* and *B. bovis*. Oliveira-Sequeira et al. (2005) reported PCR detection assays for *B. bovis* and *B. bigemina* in ticks and cattle blood. However, their *B. bigemina* primer set was directed at a *B. bigemina* genomic DNA fragment of unknown identity (GenBank accession no. S45366) and the reported BiIBN primer sequence had a mismatch in the vicinity of the 3′ end of the primer with the intended target DNA sequence in GenBank. This mismatch might be responsible for some of the false negative results reported by these authors. Although, the *B. bovis* primer set reported in their study had excellent specificity for rhoptry-associated proteins of *B. bovis*, we felt that due to the overall conserved nature of 18S rRNA genes in general and with *Babesia* specifically, the 18S gene would be a better target for our PCR assay needs and avoid false negatives due to sequence differences which may arise among the rhoptry-associated proteins as new strains of *Babesia* evolve and are identified.

During the initial developmental stages of our assay, we used multiple sequence alignment of the *Babesia* 18S rRNA GenBank entries to identify 18S regions which could meet the objectives of both the primary and nested PCRs and to analyze primer specificity *in silico*. In addition, a BlastN analysis was performed with each primer to identify sequences in GenBank with identity to the query primer sequence (data not shown). Primer KB-16 and -17 met the criteria for our primary PCR, possessing identical sequence matches for all but one of the 114 *Babesia* 18S GenBank entries. This entry was a *Babesia cf. crassa* strain and even this 18S sequence was a perfect match for all but one of the primer nucleotides and is expected to amplify under our reaction conditions. *In silico* analysis found primers KB-18 and -19 and KB -24 and -25 would be specific for *B. bigemina* and *B. bovis*, respectively. To verify these primers would maintain species specificity using samples containing related *Babesia* species, we designed the blind study with singly and mixed infected tick samples, performing both the *B. bigemina* and *B. bovis* assays separately on all samples. The specificity of both assays was verified, as all *B. bigemina*-infected samples were correctly identified in the presence or absence of *B. bovis*-infected material. Likewise, all *B. bovis*-infected samples were correctly identified in the presence or absence of *B. bigemina*-infected material. In addition, we isolated PCR product from each assay's diagnostic band and used DNA sequencing to verify the product's identity. We have also performed the PCR assay on cattle blood, using the proteinase K DNA isolation protocol on a 200 μl blood sample. We have used the rapid grind-boil procedure on unfed adult males and unfed nymphs and had satisfactory DNA yields for PCR. However, we expect lifestages containing significant quantities of host blood, especially fed adult males or engorged females, would require the more extensive proteinase K isolation protocol. We have obtained good results with the rapid crush-boil DNA protocol from engorged females when dissected cuticle fragments or hemolymph were utilized. Other species of ticks would likely require similar DNA isolation protocols as the corresponding lifestages of *R. microplus*, however, under those constraints the PCR assay should perform well.

Using the PCR assay, we verified the presence of *B. bigemina* infection in every laboratory strain of *R. microplus* maintained at the CFTRL facility. It was not possible to identify the source or timing of the original

infection, as archival material from each generation was not available for most strains. It is possible strain handling problems contributed to spreading the *B. bigemina* infection at CFTRL from one or a few tick strains to the entire strain collection.

The PCR tests on the initial collection of outbreak strain material received at CFTRL found that at least some of these ticks became infected with *B. bigemina* though natural processes, a finding which emphasizes the danger presented to the U.S. cattle industry by outbreaks of *R. microplus*. Our results have shown that engorged female ticks taken from cattle on Texas ranches (Clareno, Deutch, Las Palmas, and Valmar outbreaks) were infected with *B. bigemina* and presented a risk that cattle fever would be transmitted to U.S. cattle and reintroduced into the cattle population had these infestations of *R. microplus* not been detected and eradicated. Prevention of cattle fever in the U.S. has focused on vector (tick) control. Although the presence of the pathogen in cattle imported from Mexico should be expected, the disease need not be feared provided the success of the vector control program continues. It should also be emphasized that all outbreaks of *Boophilus* ticks in the U.S. have been eradicated by the protocols of the U.S. Cattle Fever Tick Eradication Program. Although this PCR assay is not designed to be quantitative, the level of infection in these outbreak strains likely varied, as Deutch and Las Palmas seemed to have low levels of infection because their f1 generation larvae generally produced low levels of diagnostic product in the nested assay. The Clareno and Valmar outbreak f1 larvae consistently gave strong diagnostic product bands, indicating a higher infection level.

After working with the Deutch and Las Palmas outbreak strains, which appeared to have lower infection levels, we implemented two practices when assaying new outbreak strains for *Babesia* infections. These were done solely to address the CFTRL's need to ensure that false negatives not occur in assays performed in support of the U.S. Cattle Fever Tick Eradication Program. Firstly, we run replicates of each tick strain using undiluted, 1:5 and 1:50 template dilutions in H_2O during the primary PCR. This range of template concentration ensures reliable detection of low and high levels of parasitemia within the tick. Secondly, we decreased the annealing temperature of the primary PCR by 1 °C (to 65 °C), the nested *B. bovis* PCR by 1 °C (to 65 °C), and the nested *B. bigemina* PCR by 2 °C (to 67 °C). In the *B. bigemina* assays, these changes resulted in the appearance of slightly elevated amounts of three spurious bands having molecular weights between 400 and 600 bp which are visible during the more stringent assays at the optimized annealing temperatures (Fig. 1, lanes 3 and 5). We did not identify any *B. bovis*-infected outbreak strains and when these PCR products are analyzed by agarose gel electrophoresis, no amplification products are visible. However, we did use the reduced annealing temperature protocol on *B. bovis*-infected Pullman strain template. Spurious bands of approximately 500 bp appear which are absent under the more stringent annealing temperature conditions but these bands are easily distinguished from the 217 bp diagnostic product and did not preclude accurate identification of the *B. bovis* 18S product (data not shown).

The outbreak strains are not the only possible source for the *B. bigemina* infections at CFTRL, as the Coatzacoalcos, San Alfonso, San Felipe, San Roman and Tuxpan tick strains were received from Mexico where *B. bigemina* infection is prevalent. Although frozen archival material from Coatzacoalcos was proven to be infection-free at f12 and presumably in previous generations, archival material from the original strain founding samples from San Felipe, San Roman and Tuxpan was not available to evaluate if the infection found in the current generations at CFTRL was present when received at the lab. The spread of the *B. bigemina* infection to all in-house tick strains at CFTRL was most likely facilitated by changes in the tick-rearing protocol which began several years ago as a cost-saving measure before it was known that several strains were infected with *B. bigemina*. In hindsight, a program, which screened for the presence of *Babesia* in the ticks and host animal blood would have been prudent, as would a host utilization protocol which ensured that if cattle were infested twice, the same tick strain was used for both infestations.

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